

REMARKS

The Official Action of October 18, 2005, and the prior art cited and relied upon therein have been carefully studied. Favorable reconsideration and such allowance are respectfully urged.

Claims 1-34 have been canceled in favor of new claims 35-54. Claims 35-54 remain in the application for consideration.

The Examiner has rejected claims 30, 31/30, 32/30 and 33/30 under 35 U.S.C. §103(a) as being unpatentable over Horvath '663 in view of Dee '999 or Meyer '760, and claims 30, 31/30, 32/30 and 33/30 under 35 U.S.C. §103(a) as being unpatentable over Horvath in view of Robinson '899 and either Dee or Meyer. Applicant respectfully traverses both of these rejections as applied to new claims 35-54.

Independent claim 35 now contains the feature of the membrane (4,4') having a pulling speed of 0,5 - 2 mm/day. Dee, Meyer, Robinson and Gogolewski cited by the Examiner show implants, which are not moved when implanted, i.e. which are not moved away from the vital bone for the distraction at a speed of 0,5 - 2 mm/day. Even if a person skilled in the art would have known that osteoblasts can be attached to the surface of these implants before the implantation into the

body, the person skilled in the art would not have attached the osteoblasts to a membrane of Horvath for the following reasons.

It was well known in the state of the art that there are physiological strains and hyperphysiological strains for osteoblasts. Physiological strains are around 1 000 microstrain, while hyperphysiological strains are strains of around 10 000 microstrains and higher. 1 000 microstrains correspond to a length growth of 0,1% of a cell. Since an osteoblast has a length of 10 - 30 μm , 1 000 microstrain correspond to 10 - 30 nanometres. A movement away from the vital bone for the distraction at a speed of 0,5 - 2 mm/day results in strains of 100 000 times of the physiological strain of 1 000 microstrain or 10 000 times of an already hyperphysiological strain of 10 000 microstrains per day. Therefore, a person skilled in the art would have thought that osteoblasts, which are attached to a membrane would either immediately die or at least would not give the wanted result, when a membrane is moved with a speed of 0,5 - 2 mm/day.

The documents enclosed herewith show that hyperphysiological strains i.e. strains over 10 000 microstrains lead to impaired bone regeneration:

Pages 166-172 (2001) of Meyer (Mund Kiefer GesichtsChir (5) show that hyperphysiological loads may impair bone regeneration (see abstract on page 167).

Jones et al., Biomaterials 12 (2), pages 101-110 (1991) show that the application of over 10 000 microstrains result in a dedifferentiation of the osteoblasts and a change in cell morphology to become fibroblast-like (see abstract).

Meyer et al. Int. J. Oral Maxillofac. Surg. 30, pages 522-530 (2001) show that endochondrial ossification can be found at 20 000 microstrains and is absent in samples extracted at very high strains (i.e. 200 000 microstrains). This document suggests that the histogenesis of the newly formed gap tissue is significantly determined by the applied mechanical load (see page 529, first column, 2nd paragraph).

Meyer et al., J. Oral Maxillofac Surg., 57, pages 1331-1339 (1999) shows that the application of hyper-physiologic strains results in the formation of fibrous tissue and decreases bone-forming indices.

For all these reasons, a person skilled in the art would not have attached osteoblasts on to a surface of a membrane when desiring to move the membrane with a speed of 0,5 - 2 mm/day.

Applicant has now surprisingly found that although the strain and tension applied to the osteoblasts is great and according to the expectations of the ordinary person skilled in the art would have destroyed the cells, in contrast, bone regeneration could be achieved with surprisingly excellent results. Without being limited to theory, it appears to Applicant as if the osteoblasts would, upon application of the strain, detach from the membrane so that they are not directly subjected to the strain, while simultaneously fibroblasts replace the location of the osteoblasts and these fibroblasts differentiate being subjected to the strain to osteoblasts.

Applicant respectfully submits that independent claim 35 clearly patentably defines over the cited prior art and is accordingly allowable along with claims dependent therefrom.

The prior art documents made of record and not relied upon have been noted along with the implication that such documents are deemed by the PTO to be insufficiently pertinent to warrant their applications against any of applicant's claims.

In re of Appln. No. 10/678,059
Reply dated: May 22, 2006

Favorable reconsideration and allowance are
earnestly solicited.

Respectfully submitted,

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Mechanical stimulation of osteoblasts in vitro

Abstract

Background: Mechanical loading of bone is known to play a crucial role in bone remodeling and regeneration. Whereas the clinical effects of mechanically modulated bone healing have been extensively studied, less is known about the underlying mechanisms on a cellular level. This study was aimed at investigating the effects of uniaxial strains on osteoblast-like cells in culture. Mechanical loading was applied in physiological and hyperphysiological magnitudes. Nonstimulated cultures served as controls.

Results: Cultured primary bovine periosteal cells exhibited phenotypic features of osteoblast-like cells. Application of physiological strains (2,000 μ strain) led to a bone-specific expression of extracellular matrix proteins (osteonectin, osteocalcin, collagen type I). Hyperphysiological loads (10,000 μ strain) were associated with an increased synthesis of proteoglycans. Proliferation of cells was higher than the controls at 10,000 μ strain and showed no difference from physiologically loaded osteoblasts.

Discussion: Our study demonstrates that physiological loading of osteoblast-like cells enhances the regenerative capacity of bone, whereas hyperphysiological loads may impair bone regeneration.

Keywords

Osteoblasts · Cell culture · Mechanical loading

kann, während eine mechanische Über- oder Unterbelastung zu pathologischen Reaktionen des Knochens führt [10, 11].

Die mechanische Belastung des Knochens in seiner Mikroumgebung bedeutet v. a. eine Verformung der räumlichen Struktur im Zellverband; auf zellulärer Ebene stellt eine Zelldehnung das Korrelat dieser mechanischen Belastung dar. Das Ausmaß der Dehnung (effective strain) der Knochenzellen scheint dabei die Prozesse zu modulieren, die die strukturelle Organisation des Knochens bestimmen. Klassische Beispiele, bei denen die Verformung der Knochenzellen in der Mikroumgebung von Regenerationsgewebe das klinische Ergebnis maßgeblich beeinflusst, sind die Frakturheilung und Distraktionsosteogenese. Die Mechanismen, nach denen diese „Mechanostattheorie“-abhängige Reaktion auf zellulärer Ebene funktioniert, sind allerdings noch weitgehend unbekannt [8].

Ziel dieser Studie war es daher, in einer primären Osteoblastenkultur das Verhalten von Knochenzellen auf physiologische und hyperphysiologische Dehnungsreize zu untersuchen. Die entscheidenden Parameter der Knochenzellreaktion

- Proliferation,
- Differenzierung sowie
- die Synthese knochenspezifischer Matrixproteine

wurden quantitativ bestimmt.

Material und Methode

Zellkultur

Die Zellpräparation erfolgte entsprechend einer Auswachsmethode von Jones et al. [15]. Perioststücke vom bovinen Metakarpalknochen wurden unter sterilen Kautelen entnommen und in HGEM mit 10%igem fetalen Rinder Serum (Flow Laboratories, Rickmansworth, U. K.) 4 Wochen lang kultiviert. Der Mediumwechsel erfolgte wöchentlich.

Nach Konfluenz der Zellen wurden diese mittels Kollagenase- (0,4 g Kollagenase und 98,8 mg Ham's F10 in 10 ml HEPES-Puffer) und anschließender Tyrodelösungsbehandlung (300 mg EDTA in 1000 ml NaCl-Lösung, mit 200 mg KCl, 8 g NaCl, 1 g NaHCO₃, 50 mg NaH₂PO₄ und 1000 mg Glukose) abgelöst.

Die Zellen wurden nach Aufnahme in Ham's-F10-Medium in einer Dichte von 20.000 Zellen pro cm² in 3 cm×5 cm Kulturwells, die auf Polykarbonatplatten befestigt wurden, aufgebracht und für 3 Tage kultiviert. Die Inkubation der Zellen erfolgte bei 37°C in einer feuchten Atmosphäre, die 5% CO₂ enthielt. Phasenkontrastmikroskopische Untersuchungen dienten der Kontrolle des Kulturwachstums.

Dehnungsapplikation

Eine 4-Punkt-Dehnungsapparatur diente der Applikation zyklischer uniaxialer Dehnungsreize. Die auf der Oberfläche befindlichen Zellen wurden tensilen Belastungen unterworfen, die homogen über der gesamten Kulturplatte verteilt waren. Eine Kontrolle der Dehnungswerte erfolgte mittels Dehnungsmessstreifen. Verschiedene Dehnungen

- keine Dehnung,
- 2000 μ strain und
- 10.000 μ strain

wurden bei einer Frequenz von 1 Hz über 200 Zyklen 5 Tage lang appliziert. Die uniaxiale Dehnung (in strain) errechnet sich dabei aus dem Quotienten von der durch die Dehnungsapplikation neu entstandenen und der ursprünglichen Oberflächenlänge. Dabei entsprechen 1000 μ strain einer relativen Verlängerung von 0,1%, 10.000 μ strain einer Verlängerung von 1%.

5 Tage nach Beginn der Stimulation erfolgten die Bestimmungen der Proliferations- und Differenzierungsparameter an unterschiedlichen Proben, die jeweils einer bestimmten Dehnung unterworfen worden waren.

Zellproliferationstest

Die Proliferation der Zellen wurde 3, 5 und 8 Tage nach Beginn der mechanischen Stimulation gemessen. Das Medium wurde während der Kulturperiode in 3-tägigen Abständen gewechselt. Nach Entfernung des Überstands erfolgte die Ablösung der Zellen durch 20-minütige Kollagenaseinkubation, gefolgt von einer Tyrode- sowie einer Pronasebehandlung (0,5 mg Pronase/ml Tyrodelösung). Die Zellzählung erfolgte in einem Zellzähler (Casy1). Die Bestimmung der Zellproliferation erfolgte an 4 Kultur-

plattenflächen (3 cm×5 cm) von je 3 Kulturansätzen (12 Proben).

Immunhistochemie

Die Zellkulturen wurden nach Entfernung des Mediums 2-mal mit PBS gewaschen, bevor sie in 100%igem Methanol bei -20°C fixiert und anschließend luftgetrocknet wurden.

Die Synthese der extrazellulären Matrix wurde durch eine immunzytochemische Bestimmung der knochenassoziierten Proteinexpression determiniert. Monoklonale Antiosteokalzin-, Antiosteonektin-, Anti-Prokollagen-Typ-I- (Takara Biomedical, Frankreich), Anti-Kollagen-Typ-II- (Biogenesis Ltd., Großbritannien), Anti-bone-Sialoprotein- (Immun Diagnostik, Bensheim), Anti-Kollagen-Typ-I- (BioTrend, Köln) und Antiosteopontinantikörper (R & D Systems, Wiesbaden) wurden in spezifischen Verdünnungen eingesetzt. Die Proteoglykansynthese wurde mittels Antiproteoglykanantikörpern (Chemicon, Hofheim) dargestellt. AEC (3-Amino-9-Ethylcarbazol) wurde als chromogenes Substrat bei allen Immunanfärbungen verwendet (Dako EnVision, USA).

Die quantitative Bestimmung der resultierenden Rotfärbeintensität erfolgte mittels Bildanalyse an 4 Kulturplattenflächen (3 cm×5 cm) von je 3 Kulturansätzen (12 Proben). Die angefärbten Kulturplatten wurden gescannt und das digitale Bild mit einem Bildanalyseprogramm (Easy Win 32, Herolab, Wiesloch) bearbeitet. Die Färbeintensität wurde für jedes Feld einzeln determiniert und mit einer internen Intensitätskala skaliert.

Statistik

Die Werte wurden als Mittelwerte±Standardabweichung angegeben und mittels Student-t-Test verglichen. Ein p -Wert $< 0,05$ wurde als statistisch signifikant angesehen.

Ergebnisse

Die 4-Punkt-Dehnungen der Polykarbonatplatten resultierten in einer homogenen Strainverteilung über der gesamten Kulturfläche wie mittels Mikroskop- und Dehnungsmessstreifenuntersuchung der Oberflächenverlängerung



Abb. 1 ▲ Phasenkontrastmikroskopisches Bild des subkonfluenten Monolayers unstimulierter Zellen. Die Zellen weisen die für Osteoblasten phänotypischen Charakteristika auf

auf der Zugseite der Platte gezeigt werden konnte. Die Anheftung der Zellen auf der Polykarbonatplatte führte nach 24 h Inkubationszeit zu einer subkonfluenten Monolayerkultur. Die Zellen wiesen phänotypisch alle Charakteristiken von Osteoblasten auf (Abb. 1). Sie besaßen eine polygonale Form mit radiären Protrusionen. Die Proliferation der Zellen während der Kulturperiode führte zu einer verstärkten Zell-Zell-Interaktion der dann z. T. konfluenten Kulturen. Eine Multilayerkultur ergab sich jedoch unter keinen Versuchsbedingungen. Die Ausrichtung der Zellen war sowohl bei den Kontrollen als auch den mit 2000 μstrain gedehnten Zellen randomisiert. Auch die Applikation von 10.000 μstrain führte zu keiner bevorzugten Ausrichtung der Zellen.

Die Proliferationsrate der Zellen nach 5 Tagen Stimulation (1 Hz, 200 Zyklen) war bei den stark gedehnten Zellen höher ($p=0,09$) und bei den physio-

logisch belasteten Zellen nicht signifikant abweichend ($p=0,24$) gegenüber den Kontrollen (Abb. 2).

Die immunzytochemische Darstellung (Abb. 3) der Sekretion knochenspezifischer Proteine zeigte ein z. T. statistisch signifikantes dehnungsabhängiges Verhalten (Abb. 4).

Nach 5 Tagen der Dehnungsapplikation konnte Osteonektin unter physiologischer Belastung (2000 μstrain) intrazellulär deutlich detektiert werden, während die Applikation von 10.000 μstrain die Synthese von Osteonektin reduzierte.

Ein ähnliches Zellverhalten fand sich bei Osteokalzin. Bei insgesamt reduzierter immunzytochemischer Detektion zeigte sich eine intra- und extrazelluläre Anfärbung für Osteokalzin in der Zellkultur. Physiologische Dehnungen der Zellen führten zu einer statistisch signifikanten Zunahme der Osteokalzinsekretion. Abbildung 4 zeigt, dass hö-

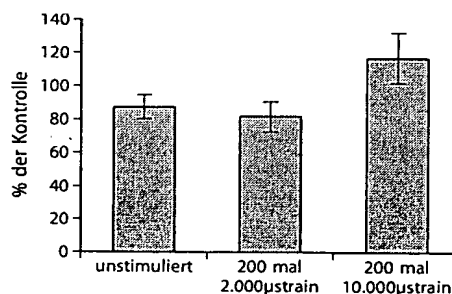
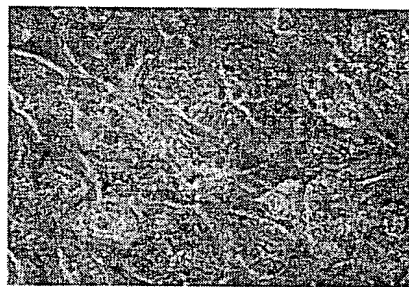


Abb. 2 ◀ Proliferationsrate osteoblastärer Zellen nach mechanischer Stimulation. Darstellung in Prozent der Kontrollen



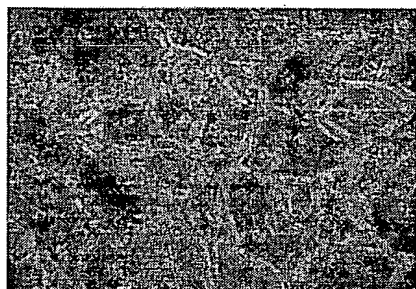
Osteokalzin Kontrolle



Osteokalzin 200x2.000µstrain



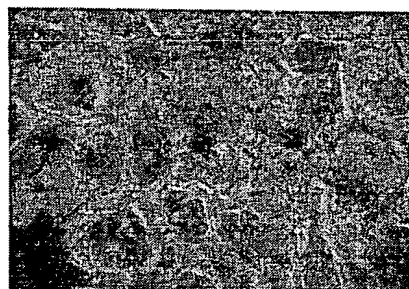
Osteokalzin 200x10.000µstrain



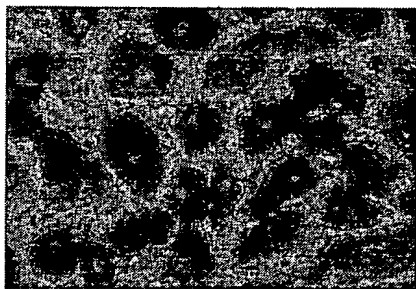
Osteonektin Kontrolle



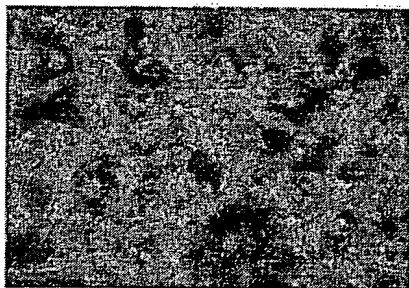
Osteonektin 200x2.000µstrain



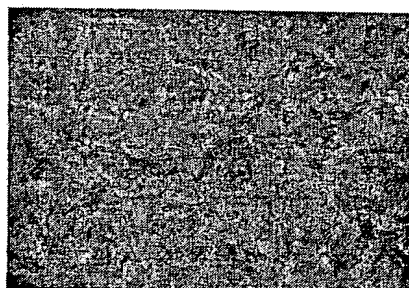
Osteonektin 200x10.000µstrain



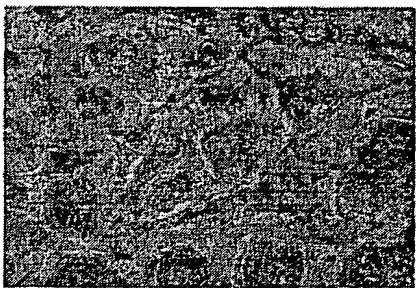
Kollagen I Kontrolle



Kollagen I 200x2.000µstrain



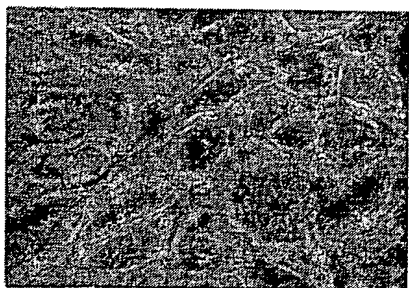
Kollagen I 200x10.000µstrain



BSP Kontrolle



BSP 200x2.000µstrain



BSP 200x10.000µstrain

Abb. 3 ▲ Immunhistochemische Darstellung der Synthese knochenassoziierter Proteine nach 8 Tagen der Stimulation (Kontrolle, 2000 µstrain, 10.000 µstrain; 1 Hz; 200 Zyklen)

here Dehnungen (10.000 µstrain) zu einer verminderten Sekretion von Osteokalzin führten, die jedoch nicht statistisch signifikant war. Osteokalzin zeigte am Ende der 5-tägigen Belastung ins-

gesamt nur eine geringe Expressionsrate. 1 Woche nach Beendigung der Dehnungsapplikation (5 Tage, 1 Hz, 200 Zyklen) war die Synthese des Osteokalzins jedoch deutlich erhöht. Bei den physio-

logisch belasteten Zellen (2000 µstrain, 1 Hz, 200 Zyklen) zeigte sich nach 1 Woche gegenüber den Kontrollen und den hyperphysiologisch belasteten Zellen eine statistisch signifikant höhere Synthe-

Originalien

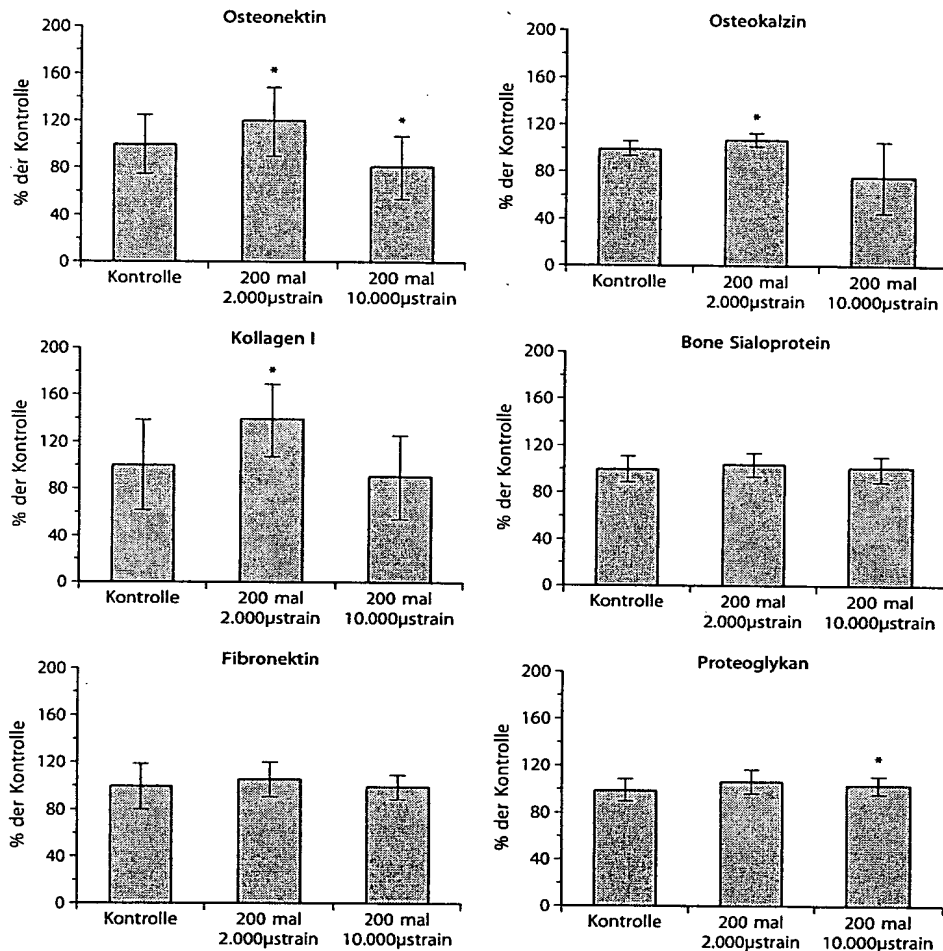


Abb. 4 ◀ Vergleich der Synthese verschiedener Knochenproteine 8 Tage nach mechanischer Stimulation (Kontrolle, 2000 µstrain, 10.000 µstrain; 1 Hz; 200 Zyklen). Darstellung in Prozent der Kontrollen sowie der Standardabweichung. Zur Kontrolle statistisch signifikante Unterschiede ($p < 0,05$) sind mit Stern gekennzeichnet

se von Kollagen Typ I. Die Synthese von Kollagen Typ I wies bei den hyperphysiologisch belasteten Zellen ähnliche Werte wie bei den unstimulierten Zellen auf. Der Mittelwert der Proteoglykan-synthese war sowohl bei 2000 µstrain als auch bei 10.000 µstrain gegenüber den Kontrollen erhöht, ein statistisch signifikanter Unterschied fand sich jedoch nur bei der Applikation von 10.000 µstrain. Die Synthese des Bone-Sialoproteins sowie des Fibronektins zeigte keine Unterschiede zwischen den verschiedenen Gruppen.

Diskussion

Sowohl verschiedene In-vitro- als auch tierexperimentelle In-vivo-Techniken wurden verwendet, um die mechanisch bedingten Osteoblastenreaktionen und deren Mechanismen näher zu charakte-

risieren [3, 4, 7, 15, 19, 26, 31]. Verschiedenartige Techniken zur Zellverformung, wie

- hypotone Schwellung,
- hydrostatischer Druck,
- die Anwendung von Flüssigkeitsscherkräften sowie
- uniaxiale Zelldehnungen,

wurden angewendet, ohne dass jeder dieser Effekte in vivo physiologisch vorkommt. Während hypotone Schwellungszustände sowie hydrostatische Druckapplikationen in vivo als eher unphysiologisch anzusehen sind, stellt eine Verformung die normale Reaktion von Knochen auf Belastungen dar.

Die Ergebnisse dieser Studie demonstrieren, dass das zyklische Dehnen von Knochenzellen in Größenordnungen von 2000 µstrain das Ausmaß der

Synthese knochenspezifischer Proteine erhöht, während die Applikation von 10.000 µstrain zu einer verminderten Syntheserate von Osteonektin bei einer erhöhten Syntheserate von Proteoglykanen führt. Während die Proliferationsrate von physiologisch stimulierten Zellen ähnlich den Kontrollgruppen ist, führt die Applikation hyperphysiologischer Dehnungen zu einer verstärkten Zellproliferation.

Die Ergebnisse dieser Untersuchung bestätigen die Ergebnisse verschiedener Autoren, die zeigen konnten, dass uniaxiale Dehnung einen bedeutsamen Einfluss auf das Proliferationsverhalten osteoblastärer Zellen sowohl in Zellkultursystemen [2, 3, 15] als auch im intakten Knochen [15, 18, 22, 29] hat.

Die in dieser Studie gefundenen, dehnungsabhängigen Veränderungen in der Proliferation der Osteoblasten wa-

ren von spezifischen Expressionsprofilen extrazellulärer Matrixproteine begleitet. Die immunhistochemische Untersuchung der Matrixproteinsynthese zeigte, dass die Syntheserate von Osteokalzin und Osteonektin bei den physiologisch gedehnten Zellen am größten war und mit steigendem Dehnungsmaß deutlich abnahm. Osteokalzin, ein Vitamin-K-abhängiges, γ -Karboxyglutamat-enhaltendes Protein, sowie Osteonektin, ein phosphoryliertes Glykoprotein, spielen sowohl in Zellkultur als auch in vivo eine essenzielle Rolle in der Knochenbildung insbesondere bei der Kontrolle der Initiation, des Wachstums sowie der Größe von Hydroxylapatitkristallen, wie verschiedene tierexperimentelle Studien zeigen konnten [14, 23, 34, 35].

Im Gegensatz zu der Untersuchung von Kaspar et al. [16] fanden sich bei unseren Studien erhöhte Expressionsraten knochenspezifischer Proteine bei physiologischen Belastungen. Dass Kaspar et al. [16] keine erhöhte Expressionsrate knochenspezifischer Proteine bei physiologischen Dehnungen fanden, mag an dem kurzen Stimulationsintervall von 2 Tagen gelegen haben, das als zu kurz für die Expression von Proteinen in Zellkultursystemen erscheint. In Übereinstimmung mit unserer Studie lag jedoch ein vergleichbares Proliferationsverhalten mechanisch belasteter Zellen vor.

In eigenen tierexperimentellen Untersuchungen zeigte sich ein dieses Studie vergleichbares Expressionsmuster von Osteokalzin und Osteonektin [20]. Die Expression von Osteokalzin und Osteonektin in einem Modell der Distractionosteogenese der Kaninchenmandibula war ebenfalls bei 2000 μ strain erhöht und wies eine deutlich verminderte Syntheserate bei 20.000 und 200.000 μ strain auf. In Übereinstimmung mit dieser In-vitro-Studie und der tierexperimentellen Arbeit sowie anderen Studien zur Knochenregeneration [17] konnte Osteokalzin als spätes Markerprotein der Knochenneubildung dargestellt werden.

Das bei hyperphysiologischen Dehnungen gefundene, veränderte Synthesemuster extrazellulärer Matrixproteine weist dabei auf Umdifferenzierungsphänomene der Osteoblasten hin. So sind Proteoglykane sowohl in der extrazellulären Matrix von chondralen Bereichen als auch in fibrösen Bereichen zu

finden [33]. In tierexperimentellen Studien konnte im mechanisch belasteten Kallusgewebe mit dehnungsabhängigen Umdifferenzierungsphänomenen das Vorhandensein sowohl chondraler als auch fibröser Bereiche nachgewiesen werden [21, 32].

Der Einfluss von quantifizierbaren Mikrobewegungen auf die Gewebedifferenzierung wurde in verschiedenen In-vitro-Studien untersucht, während die meisten In-vivo-Studien den Nachteil besitzen, dass das Verformungsverhalten nur ungenau determiniert werden kann. Eine mechanische Belastung von intaktem Knochen während des Wachstums sowie während der Frakturheilung resultierte bei vergleichbaren Studien in einer ähnlichen, von der Form der Mikrobewegung abhängigen Gewebedifferenzierung [5, 8, 12, 25]. Dabei ergab sich entsprechend den hier gezeigten Kulturergebnissen, dass eine Knochenbelastung bei physiologischen Dehnungsausmaßen zu einer regelrechten knochenspezifischen Matrixproteinsynthese führte, während höhere Dehnungsausmaße in einer fibrösen oder chondralen Gewebebildung resultierten [8, 22].

Die Ergebnisse dieser Studie verdeutlichen, dass das Ausmaß uniaxialer Dehnungen die Differenzierung und das Proliferationsverhalten von Osteoblasten determiniert. Aus klinischer Sicht erscheint die Beachtung der Belastungsfähigkeit der Zellen im Regeneratgewebe von besonderer Bedeutung. In Situationen, bei denen mechanische Belastungen und damit Mikrobewegungen auf Regeneratgewebe übertragen werden, so z. B. bei der Fixation von Frakturen, den Dehnungsapplikationen bei der Distractionosteogenese oder den Knochenbelastungen an Implantatoberflächen, sollte eine übermäßige Knochenzelldehnung vermieden werden.

Zusätzliche Untersuchungen sind jedoch notwendig, um die Art und das Ausmaß der Matrix- und Zellverformungen in dem gegenüber den Kulturbedingungen dreidimensionalen Gewebeverband unter Belastung quantitativ zu determinieren. Insbesondere die Übertragung von Extrazellulärmatrixverformungen auf die Zellverformung ist noch nicht abschließend geklärt.

Danksagung · Wir bedanken uns bei Frau Grabiniok für die sorgfältige Betreuung der Zellkulturversuche.

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Research paper:
Osteobiology

Mechanical tension in distraction osteogenesis regulates chondrocytic differentiation

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Abstract. Differentiation of chondrocytes to cells of osteoblastic phenotype occurs during an interim period of bone development, fracture repair and distraction osteogenesis. To study the relationship between tension-stress and chondrogenesis, uniaxial strains (0 μ strains, 2000 μ strains, 20 000 μ strains, 200 000 μ strains, 300 000 μ strains) were applied in a rabbit model of mandibular distraction osteogenesis. The results demonstrated that cell differentiation, apoptosis and tissue development in the newly formed gap tissue showed a correlation to the applied strain magnitudes. Only strains of 20 000 μ strains resulted in a statistically significant ($P < 0.05$) formation of cartilage struts with embedded chondrocyte-like cells. However, chondrocyte-like cells were rarely detected in samples distracted at lower or higher strain magnitudes. Osteoblasts appeared to replace cartilaginous matrix by mineralized bone matrix. The phenotypic change from chondrocytes to osteoblasts was accompanied by a decreased proteoglycan synthesis, a change in the expression from type II collagen towards type I and involved asymmetric cell divisions and apoptotic cell death. Therefore, we suggest that mechanical strain is an external stimulus responsible for phenotypic cell alterations.

Key words: chondrocytes; osteoblasts; transdifferentiation; apoptosis; mechanical strain.

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Callus formation during fracture healing is characterized by a complex pattern of cellular processes that under appropriate conditions results in the differentiation of mesenchymal precursor cells into bone-forming cells¹⁷. Distraction osteogenesis is a model of bone lengthening in which progressive distraction is the principle mechanism of new tissue formation. The histological sequence of distraction osteogenesis includes initial haematoma formation, callus formation and ultimate replacement by bone tissue. The main stages in this process are well

established and have been investigated by clinical, histological, and immunohistochemical techniques^{1,8,18,19,23,27}. Numerous investigations have demonstrated that new bone formation in distraction osteogenesis involves endochondral ossification^{21,23}. Different experimental studies described cartilaginous callus formation during the transition of soft to hard callus^{21,26,48}. Various cell types were detected in the newly formed tissue, including periosteal and endosteal lining cells, endothelial cells, marrow and immune cells,

fibroblasts, osteoclasts, osteoblasts, and chondrocytes.

It is generally accepted that chondrocytes and osteoblasts are derived from mesenchymal precursor cells, but controversy exists about the differentiation pathways during new bone formation. Some authors believe that both cell types enter separate and irreversible differentiation pathways resulting in mature and stable phenotypes^{2,20,32,36}. Recent research indicates a shared phenotypic expression of osteoblasts and chondrocytes in fracture callus¹⁷. It was shown

that endochondral bone matrix in fracture callus consisted of a mixture of bony tissue produced by osteoblasts and cartilaginous matrix produced by chondrocytes⁴³. Similar histological patterns were found in a rat model of femoral lengthening, indicating that the mode of ossification changed from endochondral to intramembranous during progression of distraction⁴². YASUI et al.⁴⁸ demonstrated that chondrocytes exposed to tension-stress switched the synthesis of collagen from cartilage-specific type II to bone-specific type I collagen. However, the exact mechanisms by which mechanical strain influences such differentiation processes *in vivo* are not clearly identified.

Previous investigations demonstrated strain-related tissue differentiation in distraction osteogenesis²⁸, however, the controversy between intramembranous versus endochondral ossification remains unclear. It is assumed that mechanical strain influences the tissue microenvironment and promotes cell differentiation in bony tissues. In the present study, we focused therefore on uniaxial strains as a putative regulator of the ossification process in the distracted callus.

Material and methods

Surgical procedures and specimen

A total of 48 adult white female rabbits, each weighing 2.6–3.4 kg, were used as experimental animals. All surgical procedures were performed as described by MEYER et al.²⁸ with aseptic technique. Rabbits were given intravenously xylazine (3 mg/kg of body weight), ketamine (20 mg/kg), and atropine (0.25 mg/kg) for anaesthesia. The lateral side of the right mandible was exposed through a longitudinal submandibular incision. A transverse osteotomy was performed on the buccal aspect immediately anterior to the first premolar using a water-cooled drill. Four self-tapping screws with a diameter of 1 mm were drilled into the mandibular body. The subperiosteal osteotomy was completed on the lingual aspect using great care to preserve the inferior alveolar neurovascular bundle and to avoid damage to the reflected periosteum. The screws were then clamped to an internal unilateral distraction device. The device was positioned along a plane perpendicular to the direction of the osteotomy (Fig. 1A). Then the periosteum was sutured to cover the osteotomy site.

The whole distraction device was inserted subcutaneously, while only the distraction axis was protruding through the skin. All animals survived the operation and were allowed to recover for 4 days. Discontinuous distraction was performed over 10 days with 0 μ strains, 2000 μ strains, 20 000 μ strains, 200 000 μ strains and 300 000 μ strains, respectively. One or 10 (2000 μ strains, 20 000 μ strains) loading cycles/day were applied (see Table 1). The lengthening procedure was performed following a calculated schedule for each strain magnitude based on the ratio between the increment of lengthening and the initial gap size. The interfragmentary strain (IFS) was calculated by the interfragmentary length (IFL) and the initial gap size (L) following the definition of strain ($\text{IFS} = \text{IFL}/L$). Different strains were applied using a gradual length increment of the distraction device. Length control was performed by intermittent plain radiographs as well as by ultrasound investigations at daily intervals. The initial gap size in all animals was 1 mm. At the end of the distraction period (10 days), the animals were sacrificed and their entire mandibles were harvested. All procedures carried out on the animals were approved by the Institutional Animal Care and Use Committee at the University of Münster, Germany.

Light microscopy was carried out on serial sections of all specimens. Briefly, mandibular specimens including the newly formed bone were sectioned perpendicular to the periosteal tube. The distraction area was identified and divided longitudinally into two halves. For the light microscopical examination, the gap tissue was fixed in phosphate-buffered paraformaldehyde. The other half preserved for electron microscopy was stored in buffered glutaraldehyde. For histological and immunohistochemical analysis, tissue samples were carefully decalcified in 10% ethylenedinitrilo-tetra-acetic acid salt dihydrate (Titrplex III, Merck) over a period of 6 weeks, embedded in paraffin and cut into sections of approximately 8 μ m thick. Sections were stained with Alcian blue for conventional histology and with a combination of Alcian blue, Alcian green and Nucleus red for cartilage staining. The non-operated mandibles served as controls. Sections stained with Alcian blue, Alcian green and Nucleus red were investigated for the presence of chondroid tissue formation. Histological analysis was performed on a Zeiss microscope.

Statistical methods

The number of chondroid positive sections was calculated as a percentage of the viewed serial sections. The differences among the various strain groups were assessed by non-parametrical one-way analysis of variance (ANOVA). For multiple comparison among the groups, we chose Scheffé tests. All analyses were carried out using the commercially available statistical software programme Statistica (StatSoft). A *P*-value of <0.05 was considered significant.

Immunohistochemistry

For immunohistochemical analysis, the mandibular specimens were fixed in 4% phosphate-buffered paraformaldehyde (pH 7.2) for 4 h at room temperature. Samples were decalcified in EDTA and embedded in paraffin. Serial sections 5 μ m in diameter were cut and mounted on slides coated with poly-L-lysine. Sections were deparaffinized with xylene and subsequently passed through a series of decreasing concentrations of alcohol into deionized water. After rinsing with Tris-buffered saline (TBS), sections were incubated with primary antibodies in moisture chambers for 16 h at 4°C. All primary antibodies were diluted in Dako Antibody Diluent (Dakopatts, Glostrup, Denmark). The monoclonal antibodies anti-osteocalcin from clone OC4-30 and anti-osteonectin from clone OSN4-2 both diluted 1:500 were received from Takara (Shiga, Japan). Monoclonal antibody anti-collagen type I from clone COL-1 diluted 1:100 was purchased from Sigma (St Louis, MO, USA), and anti-collagen type II from clone 5B2-5 diluted 1:100 was purchased from Quartett, Berlin, Germany. Prior to collagen type II staining, deparaffinized sections were demasked by hyaluronidase treatment. Hyaluronidase (Sigma, Germany) was applied in a phosphate-buffered saline solution (pH 5) at 37°C for 60 min. After incubation with primary antibody, the sections were rinsed three times in TBS. For the visualization of primary antibodies, we used the EnVision system (Dako, Hamburg, Germany, Kit. No. 1392). Briefly, sections were incubated with polymeric conjugates of soluble dextran polymers labelled with alkaline phosphatase. The chromogen reaction was stopped after 25 min and the sections were counterstained with Mayer's Haematoxylin. Negative controls with non-immune serum were also performed.

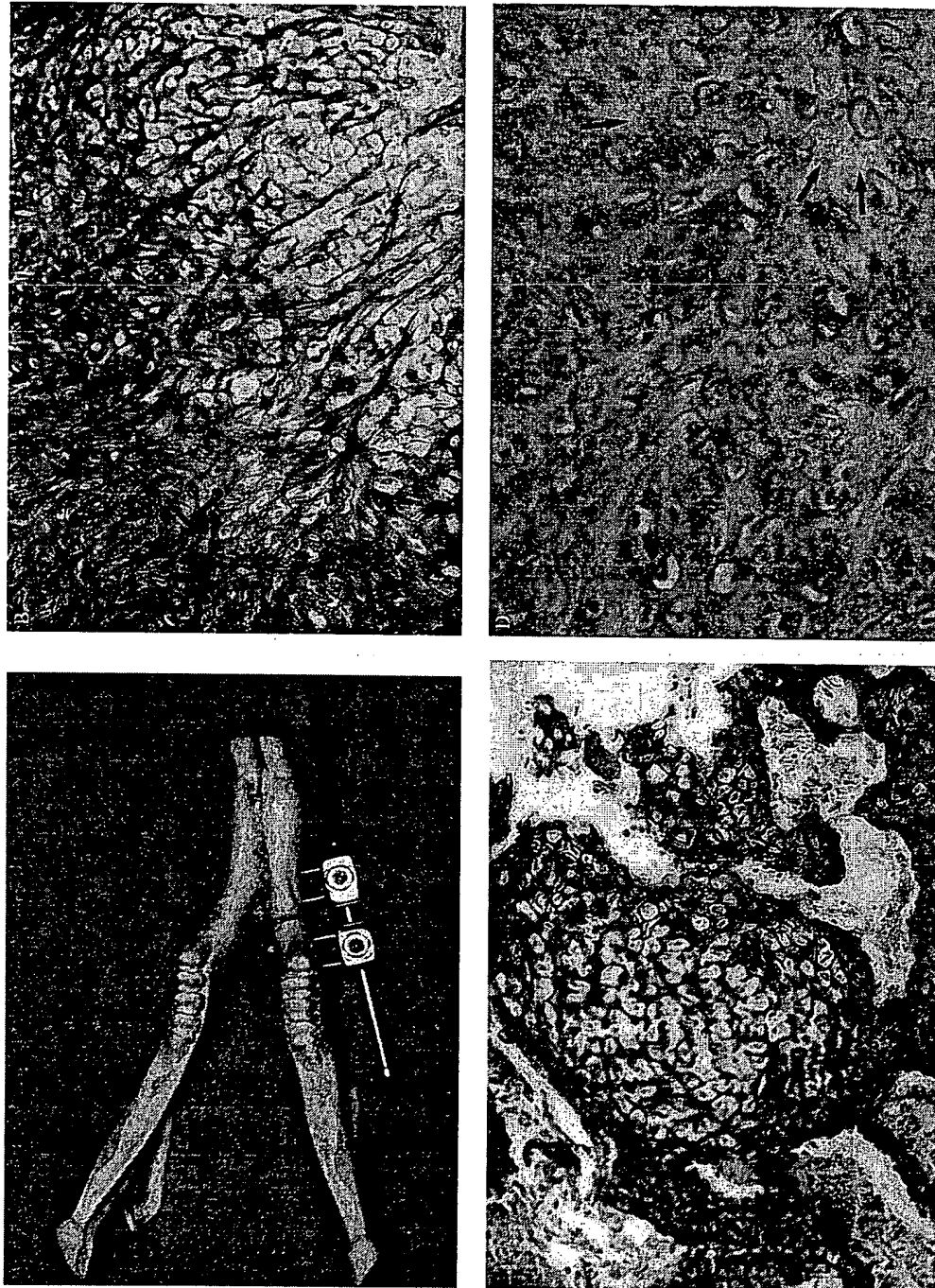


Fig. 1. (A) Scheme of mandibular distraction. (B-D) Histological appearance of callus tissue in distraction osteogenesis. Osteotomized rabbit mandibles were subjected to mechanical distraction at 20 000 μ strains with 10 cycles/day. The gap tissue between the osteotomy lines was stained with Alcian blue. (B) Low magnification of the gap tissue ($40\times$). (C) Micrograph showing cartilage struts at medium magnification ($\times 100$). (D) At higher magnification, numerous hypertrophic chondrocyte-like cells embedded in an extracellular-rich matrix (arrows) were visible. Some cells displayed typical features of osteoblasts ($\times 400$).

Table 1. Experimental schedule and results of bone lengthening

Group	No. of animals	Corticotomy	Applied strain	Bone elongation (mm)	Overgrowth of incisors (mm)	Percentage of chondrocyte-positive sections
I	6	No	—	0	-1.5	3 ± 2%
II	6	Yes	0 μ strains	0 ± 0.01	-1.5 ± 0.2	10 ± 5%
III	6	Yes	2000 μ strains	0.02 ± 0.01	-1.5 ± 0.3	15 ± 7%
IV	6	Yes	2000 μ strains	0.22 ± 0.02	-1.2 ± 0.3	17 ± 8%
V	6	Yes	20 000 μ strains	0.20 ± 0.02	-1.3 ± 0.2	62 ± 6%*
VI	6	Yes	20 000 μ strains	7.31 ± 0.2	+6.8 ± 0.6	80 ± 7%*
VII	6	Yes	200 000 μ strains	6.2 ± 0.2	+5.8 ± 0.8	7 ± 3%
VIII	6	Yes	300 000 μ strains	13.7 ± 0.4	+11.2 ± 1.5	6 ± 4%

Mean ± standard deviation, *statistical significant towards corticotomized mandibles $P < 0.05$.

Transmission electron microscopy

Mandibular samples were fixed in 4% glutaraldehyde diluted in phosphate buffer (pH 7.4). Specimens were contrasted with 1% osmium tetroxide. Samples were then dehydrated in a graded series of alcohols, embedded in Spurr's epoxy resin, and thermally polymerized at 65°C for 24 h. Specimens were sectioned on a LKB ultramicrotome. Finally, the ultrathin sections (80 nm thick) were stained with 2% uranyl acetate and Reynold's lead citrate. Sections were examined on a Philips CM10 electron microscope (Phillips, Eindhoven, The Netherlands) for electron microscopical features of cell phenotypes.

In situ detection of apoptotic cells

DNA fragmentation occurring in distraction osteogenesis as a result of apoptotic cell death was analyzed by a modified terminal transferase-mediated digoxigenin-conjugated nick end labelling (TUNEL) method, similar to that described by GENTILI et al.¹³. After washing in phosphate-buffered saline (PBS), the tissue samples were digested with 40 μ g/ml proteinase K (Boehringer, Mannheim, Germany) for 15 min at room temperature. Endogenous peroxidase activity was inhibited with 3% hydrogen peroxide in PBS followed by two rinses with PBS. Sections were washed in potassium cacodylate-containing equilibration buffer and incubated with terminal deoxynucleotidyl transferase, using the ApopTag plus *in situ* apoptosis detection kit (Oncor, Gaithersburg, MD, USA). Labelled deoxynucleotides catalytically added to the ends of DNA were detected by the use of anti-digoxigenin F_{ab} fragments conjugated to peroxidase. Sections were developed for 6 min with diaminobenzidine and counterstained with methyl

green for 5 min. Controls for the study were treated in the same way except that terminal deoxynucleotidyl transferase was substituted.

Results

Histological analysis

Previous investigations revealed that various strain magnitudes resulted in different histological patterns, as examined by conventional light microscopy^{28,31}. Areas showing endochondral ossification were rarely observed in groups distracted at low strain magnitudes (≤ 2000 μ strains) and were nearly absent in samples distracted at hyperphysiological strains ($\geq 200\,000$ μ strains) (Table 1). There was no significant difference in the number of chondrocyte-positive sections between these groups and towards the non-operated control group.

In non-distracted samples and in samples exposed to low strain magnitude (≤ 2000 μ strains), intramembranous bone formation predominated with interconnecting trabecular columns of new bone. Strain magnitudes of 20 000 μ strains resulted in small trabecular column formation that progressed centripetally into the osteotomy gap. In most of these specimens, areas of premature bone tissue and endochondral ossification were present (Fig. 1B). At 20 000 μ strains, regions of endochondral ossification were found in direct contact to columns of trabecular bone. Alcian blue staining in these areas revealed chondrocyte-like cells adjacent to osteoblastic cells. The overall appearance in each section demonstrated relatively uniform histological patterns. The mixture of chondroid and osseous tissue was statistically significantly increased ($P < 0.05$) in mandibles distracted at 20 000 μ strains (Table 1). The presence of chondrocytes was a characteristic

feature of samples distracted at this strain magnitude. The cartilage struts consisted of various phenotypic cells: chondrocyte-like cells, osteoblast-like cells, and oval-shaped cells, the morphology of which was intermediate between chondrocytes and osteoblasts (Fig. 1C,D). Often these cells were stretched along the tension vector. Cartilage staining showed chondrocytes embedded within lacunae in a basophilic chondroid matrix, whereas adjacent trabecular bone demonstrated osteoid matrix with prominent osteoblastic rimming. No osteoclasts were visible at this early stage of callus formation.

Chondrocyte-like cells were recognized predominantly in samples exposed to 20 000 μ strains, but were nearly absent at 200 000 and 300 000 μ strains. Sections of the newly formed gap tissue distracted at 200 000 and 300 000 μ strains showed primarily a soft collagenous tissue crossing the osteotomy gap with occasionally interspersed fusiform cells.

By means of immunohistochemistry, osteocalcin was identified in the extracellular bone matrix. Osteocalcin immunoreactivity was detected especially in cells of the trabecular bone surface. Chondrocytes within areas of endochondral ossification exhibited a low staining reactivity, suggesting a low osteocalcin expression or lack of incorporation into the matrix (Fig. 2A). Staining was intermediate in the transition zone of cartilage struts.

Osteoid-like tissue exhibited a positive immunoreactivity for osteonectin (Fig. 2B). Similar to the pattern of osteocalcin staining, chondrocytes within cartilage struts also displayed a low expression of osteonectin. Collagen type II was detected in regions with chondrocyte-like cells (Fig. 2C). The level of collagen type II secretion

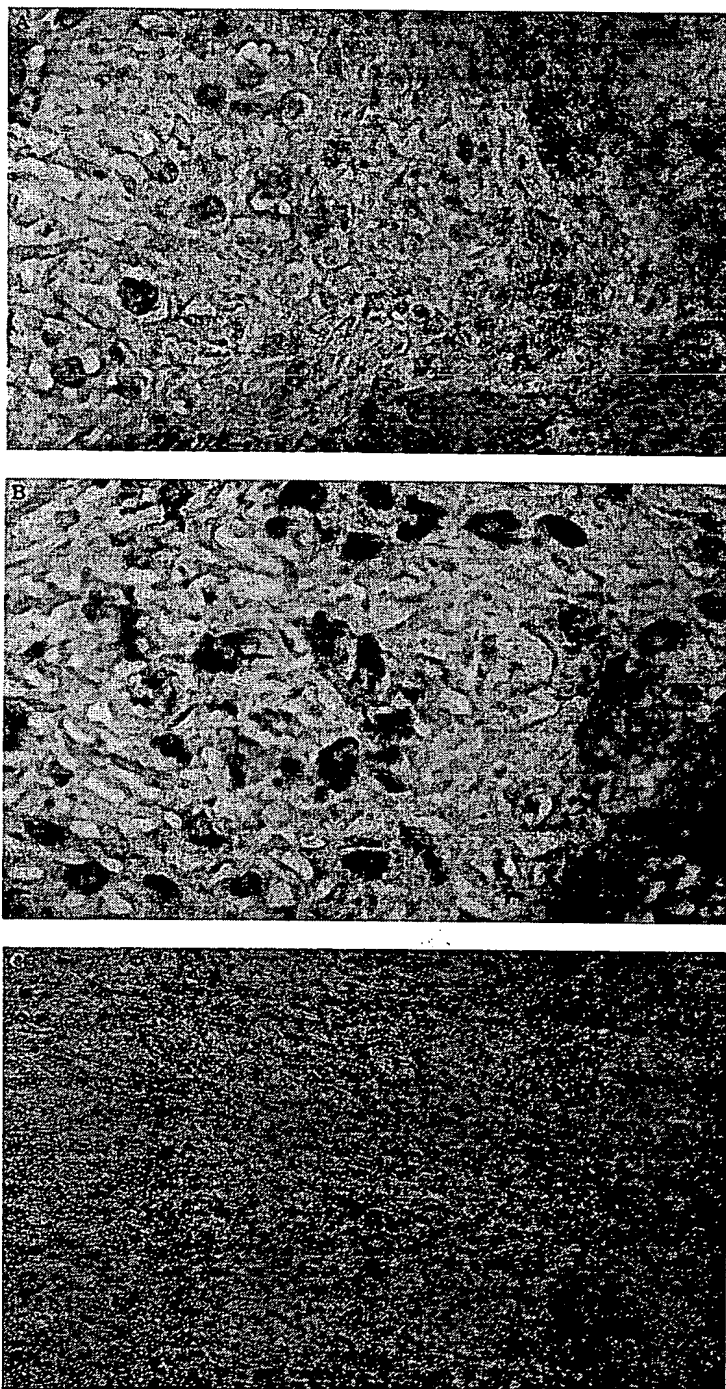


Fig. 2. Immunohistochemical detection of osteocalcin (A = $\times 400$), osteonectin (B = $\times 400$), and collagen type II (C = $\times 40$) in distraction osteogenesis. Specimens of distracted rabbit mandibles (20 000 μ strains, 10 cycles/day) were stained with the monoclonal antibodies anti-osteocalcin OC4-3 (A), anti-onectin OSN4-2 (B) and anti-collagen type II 5B2-5 (C), followed by incubation with polymeric conjugates of soluble dextran polymers labelled with alkaline phosphatase. For collagen staining, sections were pre-treated with hyaluronidase.

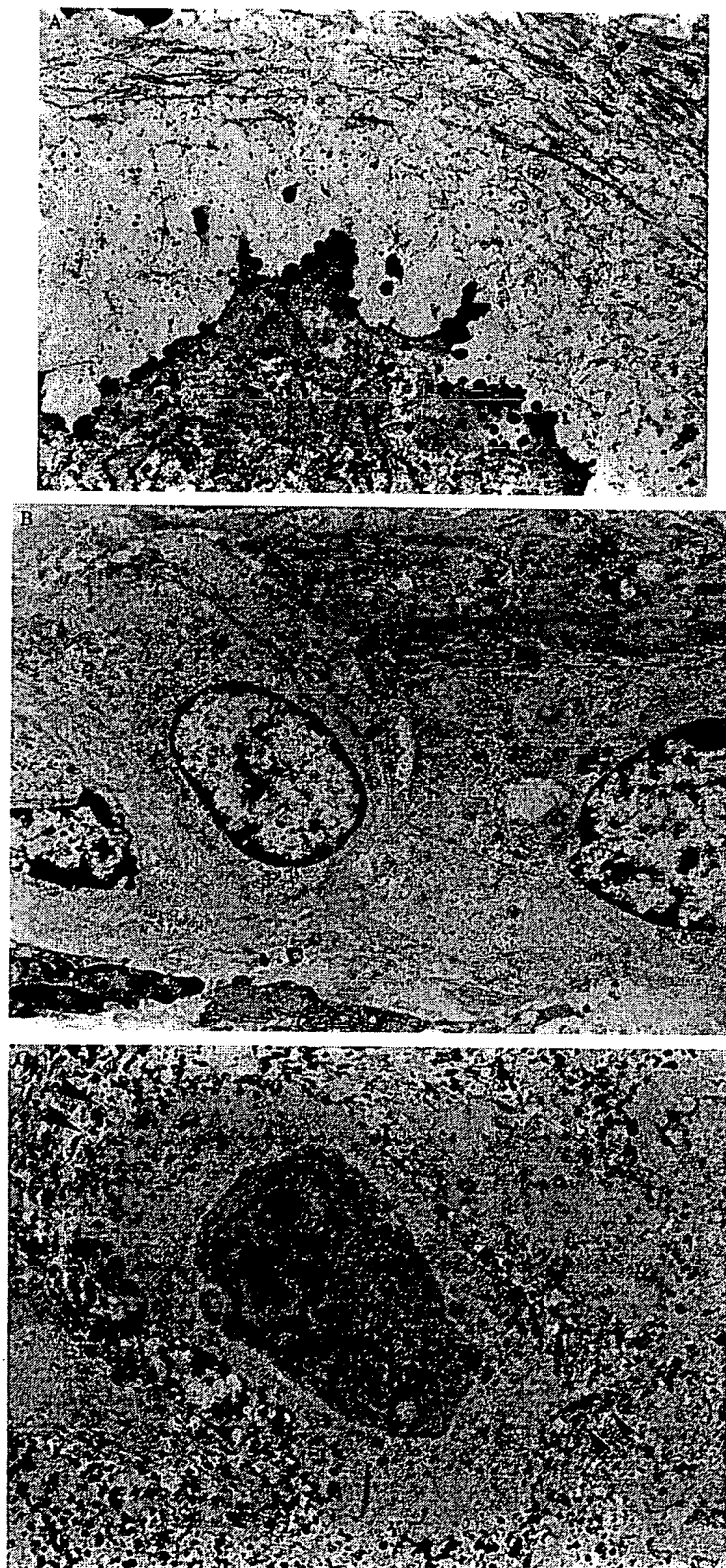
decreased in the vicinity of osteoblast-like cells. Osteoid-like tissue stained negative for collagen type II, but was positive for collagen type I.

Characteristic ultrastructural features of chondrocyte-like cells were detected in samples exposed to hyperphysiological strains. Chondrocytes were identified as large, pale cells that filled the lacunae of the chondroid matrix. Frequently, chondrocytes displayed an intense metabolic activity, as judged by large and numerous mitochondria and a developed rough endoplasmic reticulum (Fig. 3A). Chondrocytes were usually surrounded by a chondroid-like extracellular matrix consisting of numerous collagen fibres. No signs of extracellular matrix mineralization were visible. At increasing distances from the centre of the cartilage struts into the bony trabeculae, cells had an oval appearance, intermediate between chondrocyte-like cells and osteoblast-like cells (Fig. 3B). In specimens distracted at physiological strains, deposits of early mineralized matrix consisting of needle-shaped crystals were detected.

At the border to trabecular bone, some areas contained two different cell types with either a chondrocyte or osteoblast-like appearance. Cells with typical morphologic features of osteoblasts showed a relatively electron-dense cytoplasm, active endoplasmic reticulum and eccentric nuclei. These cells were usually embedded in an extracellular matrix consisting of mineralized collagen fibres (Fig. 3C).

In some specimens, cell doublets with striking ultrastructural differences were detected (Fig. 4B). Some cells exhibited an electron-dense cytoplasm and contained numerous mitochondria and an intense rough endoplasmic reticulum, suggesting that these were viable cells of osteogenic origin. Other cells demonstrated typical features of apoptotic cell death with a densely compacted cytoplasm and a blebbed cell surface with an intact plasma membrane. Frequently, these apoptotic cells were retracted from adjacent cells.

The number of apoptotic figures demonstrated a positive correlation towards the applied strain magnitude²⁹. In specimens distracted at 20 000 μ strain, apoptotic cells with a positive TUNEL-reaction were detected preferentially in the transition zone (Fig. 4A). Lacunae sometimes contained doublets with one labelled and one unlabelled nucleus. Some single chondrocytes also showed immunohistochemical evidence of



nuclear DNA fragmentation. These apoptotic cells frequently displayed light microscopical features of nuclear and cytoplasmic condensation at higher magnification.

Discussion

It is generally accepted that mechanical load influences the cellular micro-environment and has an impact on promoting and maintaining differentiated cell types in bony tissues. Several authors have shown that environmental factors strongly regulate the development of osteoblasts and chondrocytes under defined cell culture conditions^{6,22,44,45} and in animal experiments^{42,43}. However, the relationship between the applied tension-stress and cellular differentiation is still a matter of controversy.

Using a rabbit model of mandibular lengthening, the present study investigated the influence of uniaxial strains on tissue development in distraction osteogenesis. Light microscopy, immunohistochemistry and transmission electron microscopy were performed to detect cartilaginous tissue in the distraction callus.

The applied strain magnitudes and frequencies used in this experiment have been shown in previous experiments to cover the whole range of physiological and hyperphysiological strains normally found in the microenvironment of bony tissues²⁴. Over the past few years, several *in vitro* and *in vivo* studies have demonstrated that the proliferation and differentiation of bone-derived cells is dependent upon mechanical loading^{5,7,15,34,35}. Bone loading in the range between 500 and 3000 μ strains was shown to be physiological, leading to increased bone formation in the osteotomized gap tissue. Higher strain magnitudes (>5000 μ strains) known to be hyperphysiological resulted in bone

Fig. 3. Ultrastructural features of the transitional zone in distraction osteogenesis. Specimens exposed to mechanical tension (20 000 μ strains, 10 cycles/day) were examined by means of transmission electron microscopy. (A) Hypertrophic chondrocytes were embedded in a non-mineralized extracellular matrix ($\times 5500$). (B) Ultrastructural appearance of an oval-shaped cell showing morphological patterns between those of chondrocytes and osteocytes ($\times 1950$). (C) Osteoblast-like cells adjacent to a partially mineralized osteoid matrix (arrowheads) ($\times 3300$).

failure or fibroblastic dedifferentiation of cells²⁸.

Distraction osteogenesis is a principle of bone lengthening in which new bony tissue is created by progressive distraction of osteotomized bones. While it was shown by various authors that endochondral ossification is present during the process of distraction osteogenesis, there are conflicting results concerning the basic cellular events controlling the mode of ossification^{23,42}. Our previous experiments demonstrated that various tissue responses accompanied by significant different rates of apoptotic cell death are present in the early distraction phase^{28,29}. At low strain magnitudes ($\leq 2000 \mu\text{strains}$), intramembranous bone formation with interconnecting trabecular columns of new bone predominated, whereas histological sections of callus tissue exposed to hyperphysiological strains ($\geq 200\,000 \mu\text{strains}$) revealed a soft collagenous tissue crossing the osteotomy gap.

The data of this investigation indicate that intermediate strain magnitudes ($20\,000 \mu\text{strains}$) resulted in callus formation displaying typical features of endochondral and intramembranous bone formation. Round and oval-shaped cells appeared in the distraction gap and were usually arranged along the tension vector. The morphology of these cells was intermediate between osteoblasts and chondrocytes. Ultrastructurally, these cells exhibited numerous mitochondria and a developed rough endoplasmic reticulum. These cells were typically found in samples of intermediate strain magnitudes and were nearly absent in gap tissues exposed to $200\,000$ or $300\,000 \mu\text{strains}$.

A valid marker for the osteogenic phenotype at the border of trabecular bone was the presence of a mineralized bone matrix as revealed by element analysis and electron diffraction analysis³⁰, since osteogenic cells are known to produce osteoid matrix. In contrast, chondrocytes failed to synthesize an extracellular matrix capable of mineralization. In samples distracted at physiological strains, cells at the border to trabecular bone secreted collagen type I and accumulated mineralized deposits in their vicinity, suggesting that they were of an osteoblastic origin. Osteoclasts were not observed in the gap area at early stages of new tissue formation. These findings indicated that the magnitude of mechanical tension-stress clearly influenced the phenotypic differentiation of cells in the osteotomized gap tissue.

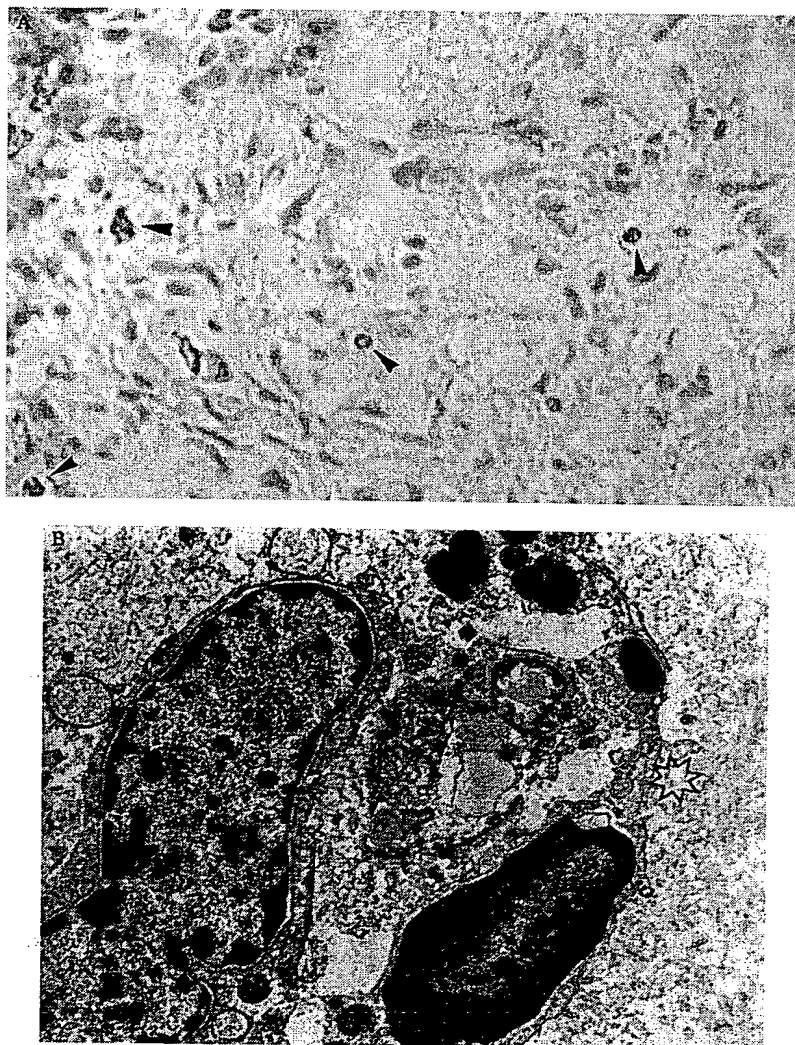


Fig. 4. Identification of apoptotic cell death in distraction osteogenesis. (A) *In situ* demonstration of fragmented nuclear DNA by a modified TUNEL method. Multiple apoptotic cells (arrowhead) were labelled immunohistochemically using the ApopTag plus apoptosis detection kit at higher strain magnitudes ($200\,000 \mu\text{strains}$, 1 cycle/day) ($\times 400$). (B) Electron micrograph showing an apoptotic cell (asterisk) in the vicinity of an osteoblast-like cell in the transitional zone ($20\,000 \mu\text{strains}$, 10 cycles/day) ($\times 3600$).

In samples exposed to mechanical strain, chondrocyte-like cells within cartilage struts at the border of trabecular bone were adjacent to osteoblast-like cells capable of producing a mineralized bone matrix. Particularly, uniaxial strains of $20\,000 \mu\text{strains}$ led to the close association between various cell phenotypes. The transition in cell phenotype and the expression of extracellular matrix proteins was demonstrated by various authors in different *in vitro* and *in vivo* studies^{11,13,14,16,33,38}. In the rat fracture callus at the junction between

cartilaginous and osseous tissue, a similar phenotypic appearance of chondrocytes and osteoblastic cells was reported¹⁷. In fracture healing, other authors have described a similar phenotypic transition of chondrocytes to osteoblast-like cells capable of producing a mineralized bone matrix^{9,10,12,21,25,39,40}. PAGE and colleagues³⁷ detected type I collagen around the chondrocyte lacunae of cartilage spicules during fracture repair. ASHHURST³ demonstrated that in some areas of endochondral ossification, the newly formed fracture matrix contained

both collagen types I and II. Chondrocytes trapped within bony stuts were shown by STAFFORD et al.⁴⁷ to express osteocalcin, a marker of osteoblastic differentiation^{11,16,33}. Recently, it has been shown that chondrocytes exposed to tension-stress switched from the expression of cartilage-specific type II to bone-specific type I collagen⁴⁸. The close local association of chondrocytes and bone-forming cells found in fracture healing, ossification of growth plates, and distraction osteogenesis has been described as a transdifferentiation process^{42,43}.

A similar expression pattern of extracellular matrix proteins suggested that both chondrocytes and osteoblasts were derived from a shared population of undifferentiated mesenchymal precursor cells. The differentiation of chondrocytes towards bone-forming cells may play a functional role in fracture healing and distraction osteogenesis^{4,41,44}. Our data suggest that the phenotypic differentiation of chondrocytes is related to the extent of mechanical tension. The finding of endochondral ossification at 20 000 μ strains and its absence in samples distracted at very low or high strains suggests that the histogenesis of the newly formed gap tissue is significantly determined by the applied mechanical load. Findings by Li et al.²⁶ support our hypothesis. Due to the study design, histology reveals a single time point finding and can therefore not evaluate a time-dependent differentiation process of single cells. Although we cannot directly measure the exact strains and stresses in the microenvironment of the distraction gap, the tissue homogeneity over the whole gap area suggests a uniform strain distribution between the osteotomy lines.

The present study demonstrates that apoptotic cell death is involved in these mechanically modulated differentiation processes. Numerous TUNEL-positive cells with DNA fragmentation and characteristic ultrastructural features of programmed cell death were detected especially in the transitional zone of endochondral bone formation. Adjacent to trabecular bone, osteogenic differentiation involved asymmetric cell divisions, resulting in two daughter cells with different cell fate: one daughter cell appears to die via apoptosis, whereas the other appeared to differentiate into an osteogenic cell. Because apoptotic cells are known to disappear *in vivo* within 1–2 h, either by autolysis or phagocytosis of neighbouring cells, the number of TUNEL-positive cells may under-

estimate the number of cells that are eliminated by apoptosis.

Although much more work is needed to investigate the molecular mechanisms of strain-related bone differentiation, the data presented identify mechanical strain as a determinant factor for chondrogenesis. Our findings and the data from the literature support the concept that changes in lineage commitment of differentiated cells occur as a result of external stimuli, such as fracture movement or deformation. A differentiation process between chondrocytes and osteoblasts may be responsible for the phenotypic findings, but cannot be validated by this study. Further studies are necessary to investigate this phenomenon.

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The effect of magnitude and frequency of interfragmentary strain on the tissue response to distraction osteogenesis.

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PURPOSE: Bone regeneration is believed to be partially controlled by the applied local mechanical strain. To test whether the magnitude or frequency of discontinuous traction regulates the tissue response, defined daily strains were applied on mandibular osteotomies using an implanted mechanical distractor. **MATERIALS AND METHODS:** Unilateral mandibular osteotomies were performed in skeletally immature rabbits (n = 36). and distraction was done by applying 2,000, 20,000, 200,000, or 300,000 microstrains once or 10 times (2,000, 20,000 microstrains) per day, respectively. Sham-operated animals (n = 6), serving as controls, underwent frame application and osteotomy without distraction. At the end of the distraction process, the newly formed tissue was evaluated histomorphometrically by the use of a well-defined scoring system of bone-forming indices. **RESULTS:** The highest bone-forming indices were detected in the osteotomized, nondistracted group and in samples exposed to a physiologic strain (2,000 microstrains). Application of hyperphysiologic strains (200,000 and 300,000 microstrains) resulted in the formation of fibrous tissue and decreased bone-forming indices. Using Kruskal-Wallis tests, a statistically significant relationship was found between the bone-forming indices and the applied strain magnitudes. Scanning and transmission electron microscopic examinations showed osteoblastic differentiation and early mineral deposition in samples distracted up to 20,000 microstrains, whereas higher strain magnitudes led to the formation of fibroblast-like cells surrounded by collagen fibrils and only slight mineralization. Multiple strain applications (10 cycles/d vs 1 cycle/d) did not alter the histomorphometric indices or ultrastructural morphology significantly but increased the amount of newly formed tissue. **CONCLUSIONS:** These results suggest that the magnitude and not the frequency of mechanical loading controls the differentiation of bone cells and the subsequent formation of bone tissue.

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Biochemical signal transduction of mechanical strain in osteoblast-like cells.

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The responses to mechanical loading of two types of osteoblast-like cells and skin fibroblasts were investigated using two new devices for applying defined and homogeneous strains to cells. The results indicate that only periosteal (bone surface) osteoblasts are sensitive to strains within the physiological range and that a specific strain mechanism is responsible. Osteoblasts derived from the haversian system and skin fibroblasts do not respond except at higher, unphysiological strains. The mechanism is located in the cytoskeleton and activates the membrane phospholipase C within milliseconds and may react to distension of a strain sensitive protein. Activation of phospholipase C can account for only some of the observed responses of bone to mechanical loading such as stimulation of cell division, increase in collagen and collagenase production. Application of over 10,000 μ strains results in a de-differentiation of the osteoblasts and a change in cell morphology to become fibroblast-like.

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